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Postconditioning Protects Against Endothelial Ischemia-Reperfusion Injury in the Human Forearm

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Background—Hypoxic cell death follows interruption of blood supply to tissues. Although successful restoration of blood flow is mandatory for salvage of ischemic tissues, reperfusion can paradoxically place tissues at risk of further injury. Brief periods of ischemia applied at the onset of reperfusion have been shown to reduce ischemia-reperfusion (IR) injury, a phenomenon called postconditioning. The aim of this study was to determine whether postconditioning protects against endothelial IR injury in humans, *in vivo*.

Methods and Results—Brachial artery endothelial function was assessed by vascular ultrasound to measure flow-mediated dilation (FMD) in response to forearm reactive hyperemia. FMD was measured before and after IR (20 minutes of arm ischemia followed by 20 minutes of reperfusion) in healthy volunteers. To test the protective effects of postconditioning, 3 cycles of reperfusion followed by ischemia (each lasting 10 or 30 seconds) were applied immediately after 20 minutes of arm ischemia. To determine whether postconditioning needs to be applied at the onset of reperfusion, a 1-minute period of arm reperfusion was allowed before the application of the 10-second postconditioning stimulus. IR caused endothelial dysfunction (FMD $9.1 \pm 1.2\%$ pre-IR, $3.6 \pm 0.7\%$ post-IR, $P < 0.001$; $n = 11$), which was prevented by postconditioning applied as 10-second cycles of reperfusion/ischemia (FMD $9.9 \pm 1.7\%$ pre-IR, $8.3 \pm 1.4\%$ post-IR, $P = \text{NS}$; $n = 11$) and 30-second cycles of reperfusion/ischemia (FMD $10.8 \pm 1.7\%$ pre-IR, $9.5 \pm 1.5\%$ post-IR, $P = \text{NS}$; $n = 10$) immediately at the onset of reperfusion. No protection was observed when the application of the 10-second postconditioning stimulus was delayed for 1 minute after the onset of reperfusion (FMD $9.8 \pm 1.2\%$ pre-IR, $4.0 \pm 0.9\%$ post-IR, $P < 0.001$; $n = 8$).

Conclusions—This study demonstrates for the first time that postconditioning can protect against endothelial IR injury in humans. Postconditioning might reduce tissue injury when applied at the onset of reperfusion by modifying the reperfusion phase of IR. (*Circulation*. 2006;113:1015-1019.)

Key Words: endothelium ■ ischemia ■ reperfusion ■ reperfusion injury ■ postconditioning

The prerequisite for salvage of viable myocardium and limitation of infarct size after an acute ischemic event is timely reperfusion.^{1,2} However, restoration of coronary blood flow carries with it the risk of further myocardial injury (reperfusion injury).³ Events occurring during reperfusion have been shown to be responsible in part for reversible (stunning) and, more important, lethal (necrosis/apoptosis) injury in animal models.⁴⁻⁷ Although the contribution of reperfusion injury to infarct size in humans is unknown, it is possible that minimizing reperfusion injury might reduce infarct size in humans.

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Modulating blood flow on reperfusion (through gradual or intermittent reperfusion) has been shown to reduce experimental infarct size.^{8,9} In particular, Vinten-Johansen et al¹⁰ have shown that a schedule of intermittent reperfusion

(termed “postconditioning”) applied at the onset of reperfusion reduces infarct size in animal models of myocardial ischemia-reperfusion (IR) injury. The protective effect of postconditioning is of a similar magnitude to that seen with ischemic preconditioning.¹¹ However, unlike preconditioning, the influence of postconditioning is restricted to the reperfusion phase of IR injury, and this has renewed interest in the reperfusion phase as a target for cardioprotection.¹²

Staat et al¹³ have recently shown that a postconditioning protocol of intermittent reperfusion reduces myocardial injury during primary angioplasty in patients with acute myocardial infarction. We have developed a model of IR injury that results in endothelial dysfunction in conduit¹⁴ and resistance vessels.¹⁵ In the present study, we used this model to determine the potential protective effects of different schedules of ischemic postconditioning against endothelial IR injury in humans, *in vivo*.

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Methods

Subjects

Forty studies were performed on 11 healthy volunteers (6 men, 5 women; mean age \pm SD 23.6 \pm 4.2 years; range 18 to 33 years). All volunteers gave informed consent. Studies were approved by the local research ethics committee and performed in a temperature-controlled laboratory (24°C to 26°C). All studies repeated in the same volunteers were at least 7 days apart.

Induction of IR

The nondominant arm was made ischemic by the inflation of a 9-cm-wide blood pressure cuff placed around the upper part of the arm to a pressure of 200 mm Hg for 20 minutes, as described previously.^{14,16}

Induction of Postconditioning

Postconditioning was induced by the application of intermittent short periods of ischemia and reperfusion on the nondominant arm early during reperfusion. After index ischemia, the arm was allowed to reperfuse for 10 seconds, after which the blood pressure cuff was inflated again to 200 mm Hg, which made the arm ischemic for 10 seconds. This deflation/inflation cycle was repeated a total of 3 times (1 minute total duration; PostC10). The effect of longer cycles of postconditioning (3 cycles of 30 seconds of reperfusion alternating with 30 seconds of ischemia) was also determined (PostC30).

Assessment of Conduit Vessel

Endothelial Function

Endothelial function of the brachial artery was assessed by flow-mediated dilation (FMD) of the brachial artery in the nondominant arm, as described previously.¹⁷ In this technique, reactive hyperemia of the forearm is used to increase blood flow in the brachial artery, resulting in brachial artery dilatation. A B-mode scan of the brachial artery was obtained in longitudinal section between 5 and 10 cm above the antecubital fossa with a 7.0-MHz linear-array transducer (spatial resolution of 0.1 mm¹⁸) and a standard Acuson XP10 system. Longitudinal, ECG-gated, end-diastolic images were acquired every 3 seconds for offline analysis. Arterial diameter over a 1- to 2-cm segment was determined for each image with the use of automatic edge-detection software (Brachial Tools, Medical Imaging Applications). Blood flow was manipulated in the brachial artery by a 7-cm-wide blood pressure cuff placed around the forearm immediately below the antecubital fossa. After 1 minute of baseline flow, the cuff was inflated to 300 mm Hg for 5 minutes and released, which resulted in a brief episode of reactive hyperemia. Brachial artery diameter changes in response to blood flow were assessed for an additional 5 minutes. Blood flow velocity was monitored continuously by pulsed-wave Doppler.

Experimental Protocols

Effect of IR on Vascular Dilator Function

To determine the effect of IR on endothelial function, FMD was assessed before ischemia (baseline) and at 20 minutes after reperfusion ($n=11$; Figure 1, protocol a). We have previously demonstrated that this protocol results in brachial artery endothelial dysfunction but does not have an effect on vascular smooth muscle function, because it did not alter the dilator response to nitroglycerin (NTG).¹⁴

Effect of Postconditioning on Endothelial IR Injury

To establish that protection against endothelial IR can be achieved by modifying reperfusion, postconditioning was induced in the same group of volunteers. Three cycles of 10 seconds of reperfusion and 10 seconds of ischemia (PostC10; $n=11$; Figure 1, protocol b) or 3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia (PostC30; $n=10$; Figure 1 protocol c) were applied immediately after index ischemia. To determine whether postconditioning needs to be applied at the onset of reperfusion, a 1-minute period of arm reperfusion was allowed before application of the 10-second-cycle

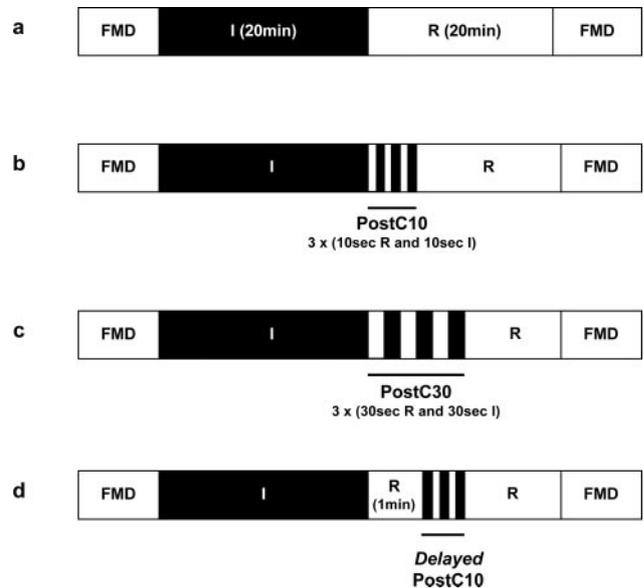


Figure 1. Protocol of studies to determine the protective effects of postconditioning against endothelial IR injury. a, FMD of the brachial artery was assessed before 20 minutes of arm ischemia (I) and at 20 minutes of reperfusion (R). To determine whether modification of reperfusion by postconditioning can result in protection against endothelial IR injury, 3 cycles of 10 seconds of arm reperfusion and 10 seconds of ischemia (PostC10; b) or 3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia (PostC30; c) were applied immediately after restoration of blood supply to the arm. To establish whether there is a requirement for postconditioning to be applied at the onset of reperfusion, the PostC10 stimulus was administered after 1 minute of arm reperfusion (DelayedPostC10; d).

postconditioning stimulus (DelayedPostC10; $n=8$; Figure 1, protocol d).

Calculations and Statistics

All data are expressed as mean \pm SE unless otherwise stated. Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter. The FMD flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume flow per minute. Data were compared by paired Student *t* test or repeated-measures ANOVA as appropriate. For multiple comparisons (8 groups), probability values by ANOVA were Bonferroni adjusted. In all cases, $P<0.05$ was considered statistically significant.

Results

All subjects tolerated the procedures without any complications. There were no differences in the responses between men and women.

Effect of IR on Vascular Dilator Function

The IR protocol had no effect on blood pressure and heart rate (Table). IR reduced brachial artery FMD (9.1 \pm 1.2% pre-IR versus 3.6 \pm 0.7% post-IR, $P<0.001$, ANOVA; $n=11$; Figure 2a), which is consistent with endothelial dysfunction. These results could not be explained by differences in brachial artery diameter (3.4 \pm 0.2 mm pre-IR versus 3.4 \pm 0.2 mm post-IR, $P=NS$, *t* test) and FMD flow stimulus (peak to baseline volume flow ratio 11.3 \pm 1.5 pre-IR versus 10.9 \pm 1.0 post-IR, $P=NS$, *t* test; Table).

Summary of Pre- and Post-IR Data From Sequential Studies in Healthy Volunteers

	IR Alone (n=11)		IR+PostC10 (n=11)		IR+PostC30 (n=10)		IR+DelayedPostC10 (n=8)	
	Pre (A)	Post (B)	Pre (C)	Post (D)	Pre (E)	Post (F)	Pre (G)	Post (H)
SBP, mm Hg	112±2	112±2	114±2	114±2	113±3	115±3	112±2	111±3
DBP, mm Hg	62±1	64±2	65±2	64±2	63±2	65±2	65±3	63±3
HR, bpm	63±2	60±3	68±3	66±3	68±4	67±4	65±3	63±3
Baseline arterial diameter, mm	3.4±0.2	3.4±0.2	3.2±0.2	3.2±0.2	3.0±0.2	3.1±0.2	3.3±0.2	3.3±0.3
Flow stimulus	11.3±1.5	10.9±1.0	8.3±1.7	8.5±1.7	8.6±1.4	7.8±1.3	8.0±1.8	8.1±1.4
FMD, % increase in diameter	9.1±1.2	3.6±0.7*	9.9±1.7	8.3±1.4††	10.8±1.7	9.5±1.5†‡	9.8±1.2	4.0±0.9*

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; and HR, heart rate.

Brachial artery endothelial function was assessed by FMD before and after IR alone (A and B), IR+PostC10 (C and D), IR+PostC30 (E and F), or IR+DelayedPostC10 (PostC10 applied 1 minute after the onset of reperfusion; G and H). Flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume flow (no units). FMD was expressed as peak % dilation from baseline brachial artery diameter.

**P*<0.001 FMD (A) vs (B) and (G) vs (H); †*P*<0.001 FMD (D) vs (B) and (F) vs (B); ‡*P*<0.001 FMD (D) vs (H) and (F) vs (H). Repeated-measures ANOVA.

Effect of Postconditioning on Endothelial IR Injury

Postconditioning had no effect on blood pressure, heart rate, arterial diameter, or flow stimulus during reactive hyperemia (Table). Ten-second-cycle postconditioning (PostC10), applied immediately at the onset of reperfusion, prevented IR-induced endothelial dysfunction (FMD 9.9±1.7% pre-versus 8.3±1.4% post-IR+PostC10, *P*=NS, ANOVA;

n=11; Figure 2b). Similarly, 30-second-cycle postconditioning (PostC30) was also protective (FMD 10.8±1.7% pre-versus 9.5±1.5% post-IR+PostC30, *P*=NS, ANOVA; *n*=10; Figure 2c). However, no protection was observed when the application of the PostC10 stimulus was delayed for 1 minute after blood flow restoration to the arm (FMD 9.8±1.2% pre- versus 4.0±0.9% post-IR+DelayedPostC10,

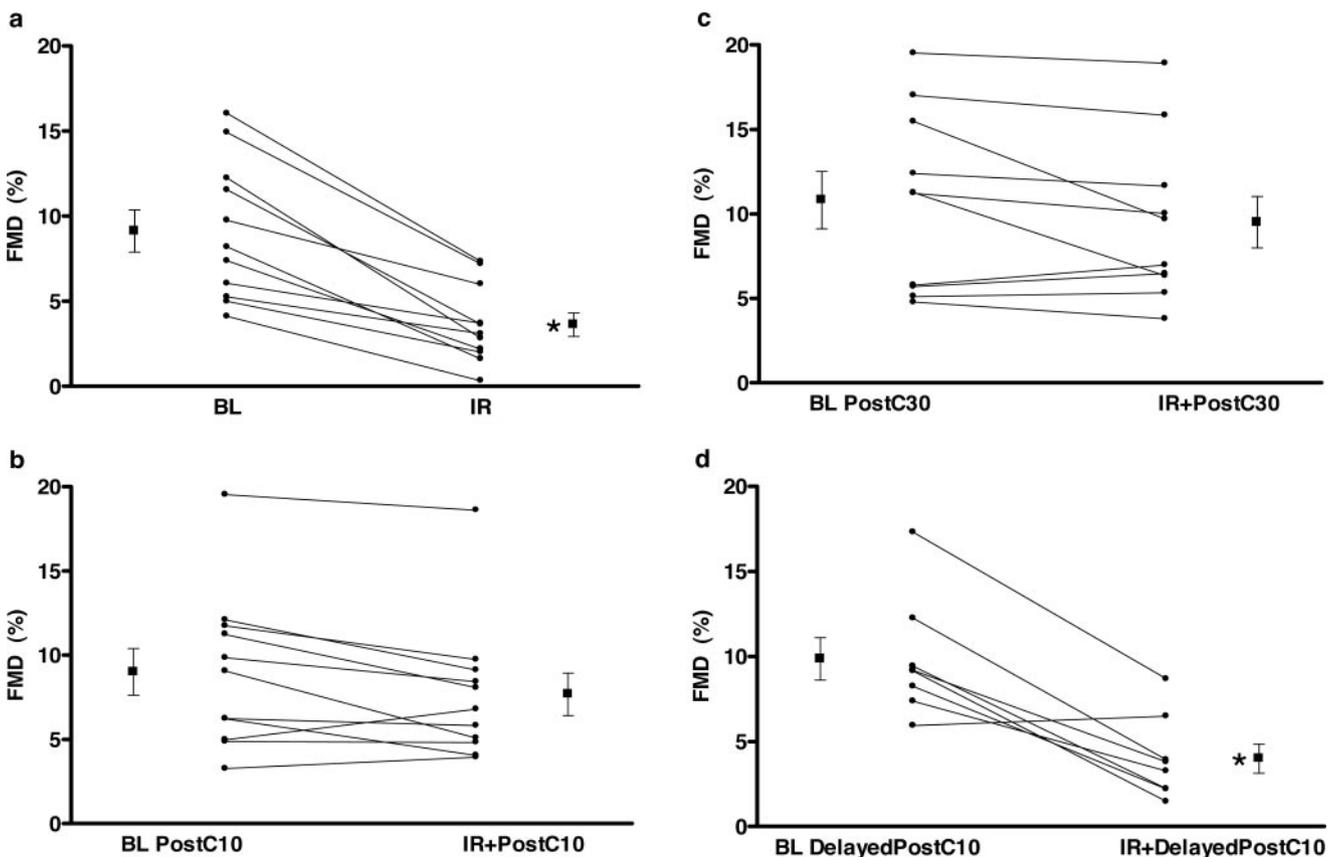


Figure 2. Effect of postconditioning on endothelial IR injury. FMD was 9.1±1.2% at baseline (BL) and was reduced by IR (a; IR 3.6±0.7%; **P*<0.001, ANOVA; *n*=11). Modification of reperfusion by 3 cycles of 10 seconds of reperfusion and 10 seconds of ischemia (PostC10), applied immediately at the onset of reperfusion, prevented the reduction in FMD (b; BL 9.9±1.7% versus IR+PostC10 8.3±1.4%; *P*=NS, ANOVA; *n*=11). A postconditioning protocol consisting of 3 cycles of 30 seconds of reperfusion and 30 seconds of ischemia (PostC30) was equally effective in protecting against endothelial IR injury (c; BL 10.8±1.7% versus IR+PostC30 9.5±1.5%; *P*=NS, ANOVA; *n*=10). Postconditioning needs to be applied at the onset of reperfusion, because no protection was observed when PostC10 was induced after 1 minute of reperfusion (DelayedPostC10; d; BL 9.8±1.2% versus IR+DelayedPostC10 4.0±0.9%; **P*<0.001, ANOVA; *n*=8).

$P < 0.001$, ANOVA; $n = 8$; Figure 2d). Mean post-IR FMD in PostC10 and PostC30 studies was significantly higher than the respective values obtained in control (IR alone; $P < 0.001$, ANOVA) and DelayedPostC10 experiments ($P < 0.001$, ANOVA; Table).

Discussion

This study demonstrates for the first time in humans in vivo that ischemic postconditioning prevents endothelial IR injury in conduit vessels. The first moments of reperfusion are critical for endothelial protection by postconditioning, because the protective effect disappeared when the stimulus was applied 1 minute after the onset of reperfusion.

Ischemic postconditioning describes protection against IR injury by a series of brief interruptions of blood flow applied at the onset of reperfusion. Studies by Zhao et al¹¹ and Halkos et al¹⁹ demonstrated in a canine model of myocardial infarction that the application of a postconditioning protocol immediately after coronary artery occlusion reduced myocardial infarct size, an effect comparable to that of ischemic preconditioning. Since then, the cardioprotective effects of postconditioning have been confirmed in the in vivo and ex vivo heart in rats^{20,21} and rabbits,^{22,23} as well as in isolated rat cardiomyocytes.²⁴ More recently, Staat et al¹³ have demonstrated that postconditioning reduces infarct size in patients undergoing primary angioplasty for acute myocardial infarction.

In the present study, we used arm ischemia to model IR injury in humans. Previously, we have shown that ischemia causes transient endothelial dysfunction of the brachial artery, with preservation of smooth muscle function.¹⁴ This model is a convenient way to compare different schedules of postconditioning stimuli. In our IR model, the degree of protection by postconditioning is similar to that achieved by ischemic preconditioning and remote ischemic preconditioning in previous studies in healthy volunteers.^{14,16} This is in agreement with animal data^{19,21} and suggests that injury suffered during reperfusion is largely responsible for the IR-induced endothelial dysfunction we observed. Moreover, our present findings demonstrate that postconditioning induces protection against endothelial IR injury by modifying events that occur during the early stages of reperfusion²⁰; a delay in the application of the postconditioning stimulus for as little as 1 minute resulted in loss of protection. Cycle length (10 versus 30 seconds) mattered less than the timing of the initiation of the postconditioning schedule. In this regard, it is worth noting that the recent study of primary angioplasty in patients used a postconditioning protocol of four 60-second cycles of ischemia/reperfusion, also initiated within the first minute of reperfusion.¹³ It remains to be determined to what extent these results in the brachial vasculature can be extrapolated to the coronary vasculature. Nonetheless, the present study provides corroborative evidence that postconditioning occurs in humans and has similar characteristics to those described in animal models and patients with acute coronary disease.

Postconditioning was initially proposed to act by reducing neutrophil-mediated damage in posts ischemic myocardium,^{11,20} but this cannot be the sole mechanism of action, because it is protective in isolated perfused hearts^{21,23} and cell

culture systems²⁴ that are neutrophil-free. There may be effects to reduce reactive oxygen species production^{19,20,25} and calcium overload.²⁴ Moreover, emerging evidence indicates that protection may be dependent on adenosine receptor stimulation,²³ opening of mitochondrial K_{ATP} channels,²² activation of the prosurvival kinases PI3K-Akt^{21,23,26} and MEK1/2-Erk1/2,²² and inhibition of mitochondrial permeability transition pore opening,²⁷ key factors implicated in ischemic preconditioning-induced protection. These observations suggest that postconditioning and ischemic preconditioning have common signaling pathways, which may explain why these protective phenomena are equally effective in protecting against IR injury.^{11,19,21}

In humans, preservation of FMD by postconditioning is consistent with increased nitric oxide availability during reperfusion, either due to decreased nitric oxide inactivation^{11,24} and/or increased nitric oxide synthesis.²¹ In the present model, IR-induced endothelial injury resolves spontaneously within 60 minutes of reperfusion,¹⁶ consistent with "endothelial stunning" rather than necrosis in response to 20 minutes of ischemia. This may explain the complete abrogation of endothelial dysfunction caused by postconditioning that we observed. Whether the postconditioning schedule we used will protect from more substantial endothelial injury (as would occur in the clinical setting of acute myocardial infarction) or be protective in patients with preexisting endothelial dysfunction (as occurs in patients with risk factors for atherosclerosis) remains to be determined.

In summary, our data demonstrate an effect of postconditioning to prevent endothelial IR injury in the human forearm vasculature. The data indicate that much of the endothelial dysfunction caused by sublethal IR injury occurs during reperfusion. Moreover, mechanisms activated in the immediate phase of reperfusion are necessary for endothelial IR injury. These data support the idea that the reperfusion phase of IR injury is a legitimate therapeutic target. Given the increasing role of primary intervention in the treatment of acute coronary syndromes, postconditioning schedules or pharmacological manipulation may modify reperfusion injury and reduce infarct size sufficiently to improve outcomes.²⁸

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The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Disclosures

None.

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CLINICAL PERSPECTIVE

This study demonstrates that intermittent reperfusion (postconditioning) after a period of ischemia prevents endothelial injury in the human forearm vasculature. It indicates that much of the endothelial dysfunction caused by sublethal ischemia-reperfusion injury occurs entirely during reperfusion. Moreover, it would appear that pathophysiological mechanisms activated in the immediate phase of reperfusion are necessary for endothelial ischemia-reperfusion injury. The results of this study are convergent with the effects of postconditioning in animal models and in one recent clinical study of patients undergoing primary coronary angioplasty. Postconditioning reperfusion schedules or timely pharmacological manipulation during reperfusion have the potential to modify reperfusion injury in the clinical setting. Whether this be primary coronary intervention, bypass, or transplant surgery, it is hoped that reduction of early reperfusion injury after prolonged ischemia will improve clinical outcomes.